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Characterization of redox-related soil microbial communities along a river floodplain continuum by fatty acid methyl ester (FAME) and 16S rRNA genes

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ABSTRACT

Redox states affect substrate availability and energy transformation, and, thus, play a crucial role in regulating soil microbial abundance, diversity, and community structure. We evaluated microbial communities in soils under oxic, intermittent, and anoxic conditions along a river floodplain continuum using fatty acid methyl ester (FAME) and 16S rRNA genes-based terminal-restriction fragment length polymorphism (T-RFLP) bacterial fingerprints. In all the soils tested, microbial communities clustered according to soil redox state by both evaluation techniques. Bacteria were dominant components of soil microbial communities, while mycorrhizal fungi composed about 12% of the microbial community in the oxic soils. Gram-positive bacteria consisted >10% of the community in all soils tested and their abundance increased with increasing soil depth when shifting from oxic to anoxic conditions. In the anoxic soils, Gram-positive bacteria composed about 16% of the total community, suggesting that their growth and maintenance were not as sensitive to oxygen supply as for other microbes. In general, microorganisms were more abundant and diverse, and distributed more evenly in the oxic layers than the anoxic layers. The decrease in abundance with increasing oxygen and substrate limitation, however, was considerably more drastic than the decrease in diversity, suggesting that growth of soil microorganisms is more energy demanding than maintenance. The lower diversity in the anoxic than the oxic soils was attributed primarily to the differences in oxygen availability in these soils.

1. Introduction

It has long been recognized that redox states play a crucial role in regulating microbial activity and community structure (Lüdemann et al., 2000). Shifting redox states from the oxic surface layers to anoxic subsurface zones leads to development of microhabitats and microbial communities stratifying along the soil profile. Microbial species in the subsurface soils could be strikingly different from those in the surface soil (Crocker et al., 2000; Fritze et al., 2000; Blume et al., 2002). Following characterization of 40 strains of G+, aerobic, heterotrophic bacteria isolated from saturated subsurface lacustrine, paleosol and fluvial sediments, Crocker et al. (2000) found that most of the subsurface isolates did not cluster with previously established surface soil species in phylogenetic analyses of 16S rRNA gene sequences or with hierarchical cluster analyses of cellular fatty acid profiles.

Although extensive studies have been conducted to reveal microbial communities in surface soils or sediments (Kieft et al., 1997; Sahm et al., 1999; Urakawa et al., 2000; Agnelli et al.,

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2004; Sun et al., 2004;), few have been directed to characterize microbial communities in the transitional redox zones that encounter alternating oxidation–reduction. Such soil ecosystems are prominent in floodplains. As the transformer, sink, source of nutrients, floodplains provide a unique set of various habitats for diverse biota (Tockner et al., 1999). Periodic erosion and sedimentation processes often lead to remobilization of nutrients, heavy metals, pesticides, and other potential environmental contaminants (Walling et al., 1996; Krüger et al., 2005). Some of these processes can have adverse effects on water quality, aquatic habitats, and the agricultural use of the floodplain area (Krüger et al., 2005; Noe and Hup, 2005).

The floodplain ecosystem of Tisza River in Hungary offers a unique opportunity in the evaluation of redox-related soil microbial communities. Tisza River has a history of periodically flooding. In 2000, 100,000 m^3 of wastewater containing cyanide and heavy metals from an upstream broken tailing dam were released to the river ecosystem (Fleit and Lakatos, 2003), jeopardizing the sustainability of the ecosystem as the home for many unique florae and faunae in Europe. Subsequently, considerable international attention has been directed to evaluate the Tisza River ecosystem health and function.

In another related study (Unpublished data), we found that the overall changes in ecosystem health and function are gradual, making it challenging to reveal changes that were directly induced by metal contaminations even though the major accidental contamination in 2000 resulted in dead fish floating in the river. Over the years, contaminants were suspended, settled, resuspended, and resettled along the river channel and floodplain. Metal concentrations in the floodplain soils taken in 2003 were relatively low compare to other reported metal contaminated soils (Pennanen et al., 1996; Konstantinidis et al., 2003; Krüger et al., 2005). For this study, the main goal was to determine impact of redox states on microbial community diversity and structure throughout the soil profile using fatty acid methyl ester (FAME) analysis and 16S rRNA-based terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting. In addition, variations in microbial communities between surface soils and between subsurface soils were also examined.

2. Materials and methods

2.1. Soils

The Tisza river region is located in central Europe with an annual precipitation of 640 mm and temperatures varying from −28 to 22 °C (10–11 °C by average). Soil samples were taken along the Tisza River in October 2003 from two locations, upstream (U, 48°13′N 22°33E for US1) and downstream (D, 48°11′N 21°44E for DS1), which represent two landscape replications. Soil profiles from both locations show hydromorphic properties and these soils are classified as Meadow-Chernozems (FAO-ISRIC-ISSS, 2006). At each location, four sampling sites (S) within a radius of approximately 1 km were identified. Sites 1–4 were defined from the river channel towards the floodplain, with their flooding recurrence interval ranging from 1 year to 100 years. At each site, a soil core (5 cm diameter and about 280 cm long) was obtained and was segmented into three samples based on redox states, including oxic (rarely waterlogged), intermittent (seasonally waterlogged), and anoxic (permanently waterlogged) layers. The redox state of the soil was determined based on redoximorphic features (Schoeneberger et al., 2002). Generally speaking, the redox zones had two chroma matrix colors; while the reduced zones showed gray depletions in sand and had 0 chroma color in clay. The anoxic layer for soil US3 was not reached following sampling to 281 cm. Field-moist soil samples were stored and transported on ice. Samples were sieved (<2 mm) and mixed thoroughly, and stored in sealed plastic bags at 4 °C. A portion of each sample was air-dried and another portion was freeze-dried. Field-moist soils were used

Table 1 – Properties of soils under redox state specified[a]

Sample site[b]	Depth (cm)			pH			Texture (%)						C_{org}(%)		
	Oxic	Int	Anoxic	Oxic	Int	Anoxic	Sand			Clay			Oxic	Int	Anoxic
							Oxic	Int	Anoxic	Oxic	Int	Anoxic			
DS1	0–30	31–180	181–270	6.3	7.3	7.1	8.4	13.7	49.6	38.8	34.8	16.0	2.99	0.83	0.42
DS2	0–127	128–180	181–280	7.2	7.3	6.8	42.1	7.7	17.9	14.6	31.7	21.9	0.59	0.90	0.29
DS3	0–74	75–125	126–280	7.1	6.8	6.7	4.0	3.5	9.7	32.6	47.0	48.2	1.46	1.09	1.13
DS4	0–95	96–184	185–270	7.4	7.9	7.9	11.4	34.9	49.9	30.1	15.0	16.7	1.25	0.24	0.35
US1	0–107	108–198	199–261	7.4	7.5	7.6	7.0	62.8	68.0	33.2	11.5	9.1	1.37	0.68	0.37
US2	0–145	146–195	196–255	7.4	7.5	7.6	55.1	74.6	75.0	12.9	8.0	7.5	0.70	0.31	0.72
US3	0–85	86–281	ND	7.2	7.0	ND	6.9	22.3	ND	33.1	22.8	ND	1.49	0.75	ND
US4	0–128	129–188	189–277	7.5	7.7	7.7	13.7	15.5	13.8	24.4	18.6	19.0	0.75	0.26	0.31

[a] D-Downstream location, U-Upstream location, S-site, Int-Intermittent, and ND-not determined.

[b] DS1 was a pasture, located in the midway between the river and the boundary to the first terrace. It is in the local low point of the floodplain. DS2 was a cultivated field located on natural levee along Tisza River. DS3 was a pasture, located near an oxbow that lies at the boundary between the floodplain and first terrace. DS4 was a pasture located on first terrace above the floodplain that has a flooding recurrence interval of 100 years. US1 was a pasture located along an oxbow drainageway near the boundary between the floodplain and first terrace. US2 was planted with hardwood trees and located on a high area of the general landscape in the floodplain. US3 was a cultivated field located about 100 m from Tisza Rive channel, not far from US1 and US2. US4 was a apple orchard located on first terrace above the floodplain that has a flooding recurrence interval of 100 years.

for biochemical and microbiological analyses. Freeze-dried soils were used for FAME and DNA analyses. Organic C content (C_{org}) was determined using air-dried samples with particle size <180 μm. All other analyses used samples that were <2 mm.

Selected basic characteristics, including soil texture, organic matter content, and soil pH, are summarized in Table 1. Loess is the most common soil parent material in the Tisza River basin. The majority of the sediments in the floodplain were silts and clays derived from within the basin. Horizons with sandier textures were present in deep (160+ cm) soil profiles. Sand contents varied from 4 to 75% and clay contents varied from 7.5 to 48%. With a few exceptions, clay contents were higher in the oxic than the intermittent and anoxic layers. Soil pH ranged from 6.3 to 7.5, 6.8 to 7.9, and 6.7 to 7.9; and C_{org} ranged from 0.59 to 2.99, 0.24 to 1.09, and 0.29 to 1.13 in the oxic, intermittent and anoxic layers, respectively. Contents of C_{org} were by average higher in the oxic layers than the intermittent and anoxic layers. Most of the soil profiles had thick A horizons and high organic matter content, which are typical characteristics of Chernozems. However, the soils were mixed based on redox state, resulting in relatively low organic matter content in some of the oxic soils. Concentrations of six metals were determined. Cd ranged from 0.7 to 2.8 mg kg^{-1} soil; Ni from 11 to 37 mg kg^{-1} soil; and Zn from 53 to 310 mg kg^{-1} soil. Concentrations of Cu, Cr, and Pb all ranged from 11 to 80 mg kg^{-1} soil. In general, total metal concentrations decreased with increasing soil depth and/or the proximity to the river channel. Metal concentrations in the upstream and downstream sites were not significantly different. Based on comparison among sites of different flooding frequency, chronic flooding and accidental heavy metal contamination reduce microbial metabolic activity, but did not lead to detectable changes in microbial biomass. Periodic flooding and oxygen limitation led to the development of microbial communities with reduced C use efficiency (Unpublished data).

2.2. FAME analysis

Fatty acids were extracted from the soils using the procedure described for pure culture isolates as previously applied for soil analyses (Cavigelli et al., 1995; Sasser, 1990; Acosta-Martínez et al., 2003). Briefly, the method consists of four steps: (1) saponification of fatty acids by treating the soil (3 g of <2 mm freeze dried soil) with 3 ml 3.75 M NaOH (methanol:water, 1:1) solution at 100 °C for 30 min; (2) methylation of fatty acids by treating the samples with 6 ml of 6 M HCl in aqueous methanol (1:0.85) at 80 °C for 10 min; (3) extraction of fatty acids methyl esters (FAMEs) with 3 ml of 1:1 hexane: methyl-*tert*-butyl-ether solution and rotating the samples end-over-end for 10 min; (4) washing of the organic phases with 3 ml of 1.2% diluted NaOH by rotating the tubes end-over-end for 5 min. The organic phase (top phase), containing FAMEs, was analyzed in a 5890 Gas Chromatograph series II (Hewlett Packard, Wilmington, DE). Peaks in a sample were compared to standard fatty acids (Microbial ID, Newark, DE) and their relative peak areas (percentages calculated against total detected areas) were determined with respect to other fatty acids in a sample using the MIS Aerobe method of the MIDI system.

The FAMEs were analyzed by the Microbial Identification System (MIS, Microbial ID, Inc., Newark, DE). FAMEs are described by the number of C atoms, followed by a colon, the number of double bonds and then by the position of the first double bond from the methyl (ω) end of molecules. Isomers *cis* and *trans* are indicated by c and t, respectively. Branched fatty acids are indicated by the prefixes *i* and *a*, for *iso* and *anteiso*, respectively. Other abbreviated notations are *Me* for methyl and *cy* for cyclopropane. The total number of peaks (showing >0.1%) and peak area of FAME detected were summarized to indicate microbial diversity and abundance.

Within the FAME profiles, individual FAME markers were investigated to compare the relative abundance of specific microbial groups. The relative abundance of Gram-positive (G^+) bacterial populations was obtained from FAME markers 15:0, *a* 15:0, *i* 15:0, *i* 16:0, *a* 17:0, and *i* 17:0; whereas that of Gram-negative (G^-) bacterial populations from cy17:0 and 18:1ω7c (Wright, 1983; Walling et al., 1996; Zelles, 1997). Actinomycetes (Actino.) abundance was obtained from 10Me16:0, 10Me17:0, and 10Me18:0 (Zelles, 1997). The FAME marker (20:4ω6c) was used for the evaluation of protozoan abundance (Walling et al., 1996). The relative abundance of fungal populations was obtained using 16:1ω5c, 18:1ω9c, 18:2ω6c, and 18:3ω6c (Wright, 1983; Frostegård et al., 1993). Total saturated/total monounsaturated fatty acid ratios (12:0 + 13:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0)/ (14:1ω5c + 15:1ω6c + 16:1ω7c + 16:1ω5c + 17:1ω9c + 18:1ω9-c + 18:1ω7c) and cyclopropyl fatty acids/monoenoic precursors (cy17:0 + cy19:0/16:1ω7c + 18:1ω7c) were calculated to evaluate the nutritional stress in bacterial communities (Guckert et al., 1986; Zelles et al., 1994; Kieft et al., 1997; Fierer et al., 2003).

2.3. DNA extraction and PCR amplification

Soil DNA was extracted using an UltraClean™ Soil DNA kit (MoBio Laboratories, Inc., Solana Beach, CA, USA). Prior to PCR amplification, DNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Fragments of 16S rRNA genes were amplified by polymerase chain reaction (PCR). Two PCR primers, BF8 and 926R (BF8 5′-AGAGTTTGATCCTGGCTCAG-3′ and 926R 5′-CCGTCAATTCC-TTTRAGTTT-3′) were used to amplify an internal region (927 bp) of 16S rRNA genes in the Bacterial Domain (LaMontagne et al., 2003). BF8 was fluorescently labeled with IRDye™ 700 at the 5′ end. Each 100 μl PCR mixture contained 25 ng of soil DNA extract, 0.5 μM of each primers, 500 μM of PCR nucleotide mix (Fisher Bioreagents, Fisher Scientific, Inc., Pittsburgh, PA), 1.75 mM $MgCl_2$, 1.5 g bovine serum albumin and 2.5 Units Taq DNA polymerase in PCR buffer A (Promega Chemicals, Madison, WI). PCR reactions were conducted at 94 °C for 2 min, followed by 94 °C for 1 min, 52 °C for 1 min and an extension at 72 °C for 2 min for 31 cycles and a final extension at 72 °C for 10 min.

2.4. T-RFLP analysis

Approximately 3 μg of fluorescently labeled PCR products were digested by a restriction enzyme at 37 °C for 5 h. Five restriction enzyme digestions were conducted, including AluI,

HaeIII, HhaI, HpaII, and RsaI (Invitrogen Corporation, Carlsbad, CA). Each digested product (20 μl) was denatured by heating at 95 °C for 5 min and then immediately quenched on ice. Aliquots (1.0 μl per well) were loaded onto a 6.5% denaturing polyacrylamide gel (urea as the denaturant) and DNA fragments were separated by electrophoresis for up to 5 h at 2,500 V and 40 mA. The sizes of the terminal restriction fragments (TRFs) were estimated by reference to internal standards of 50–700 bp.

Banding patterns of three independently repeated analysis, including DNA extraction, PCR amplification, and T-RFLP fingerprinting, were analyzed by GeneProfiler database manager with the TreeCon software (GeneProfiler, Version 4.01, Scanalytics, Inc., Fairfax, VA). The lower limit for band detection was set by the band peak height threshold of 0.1% of the total detected optical density in the lane. Each sample was scored based on the presence or absence of each band in its profile compared with the profile of each of the other samples. The total number of bands was determined to indicate total number of bacterial ribotypes. Shannon diversity indices (H′) (Shannon, 1948; Stahl et al., 2002) were calculated to reflect species richness and evenness of the bacterial community using the following equation:

$$H' = -\sum[pi(\log pi)]$$

where *pi* represents % of the integrated density of a band, relative to the sum of all the bands in a lane (GeneProfiler, Version 4.01, Scanalytics, Inc., Fairfax, VA). Species evenness of the bacterial community was also indicated by the Pielou's diversity (J) (Pielou, 1966), which was calculated using the following equation:

$$J = \frac{H'}{\log(\text{total number of bands in a lane})}$$

Similarity among the microbial communities was assessed by pair-wise matching, in which matching bands were identified by comparing two samples at a time. The band sharing coefficient (S; also termed the Dice similarity coefficient) (Dice, 1945) was used as a measure of similarity between two treatments and was determined from the following equation:

$$S = \frac{2Ns}{NT}$$

where *Ns* is the number of shared bands in samples A and B, *NT* is the total number of bands in samples A and B. Relatedness of bacterial communities was evaluated by determining similarity coefficients for bands common to two samples. Two bands were common if they migrated the same distance on a gel. Based on the number, intensity values, and positions of detected bands, cluster analyses and statistical analyses were made. Dendrograms were constructed from ''0/1''string data using Gene Profiler 4.01 software (Scanalytics, Inc., Fairfax, VA). Bands were marked as matching if the molecular weight (MW) of one of the bands was within the upper and lower MW limits of the other bands. The upper and lower MW limits of a band were determined by multiplying its MW by $1 \pm$ match tolerance. The dendrograms were created with 100 iterations, with bootstrap values providing a confidence level for each branch point. The higher the bootstrap value, the more reliable is the measurement of the branch point value.

2.5. Statistical analysis

Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was done using the least significant difference (LSD) test at a *P*-value of <0.05. Percentage data were normalized by arc sine transformation before analysis (Gomez and Gomez, 1984). Indicator FAMEs of bacterial, actinomycetes, fungal, and protozoa groups were evaluated with the PC-ORD statistical software (Version 4) using Principal Component Analyses (PCA) to determine differences in the microbial community composition of the soils tested (McCune and Mefford, 1999). A soil that clustered near a FAME vector line suggested that this soil contained a relative high abundance of the microbial group represented by this vector line. This type of PCA was performed using cross-products matrix with variance/covariance centered and calculating scores for FAMEs by weighted averaging.

3. Results

3.1. FAME analysis

Among the 122 FAMEs possibly named with the MIDI method, 99 fatty acids were extracted and detected from the soils tested. Of the 99 FAMEs identified, 39 were present in at least 20 of the 23 soils tested. The average number of FAMEs in the oxic soil layers was 58, which was significantly higher than those in intermittent (40) or anoxic soil layers (29) (Table 2). Within the same redox state, significantly lower numbers of FAMEs were detected in the site 4 soils. With a few exceptions, similar trends were shown by FAME peak areas that indicate microbial abundance. However, there was a more drastic decrease in the FAME peak area than in FAME numbers from oxic to intermittent and to anoxic soils along the soil profile. By average, the oxic soils had 1.2 FAMEs per 1000 unit peak area, while the intermittent soils had 4.3 and the anoxic soils had 6.2 FAMEs per 1000 unit peak area (data not shown), showing an increase in detected FAME numbers per unit amount of fatty acid detected.

Of the FAMEs detected, 41 of them comprised 92.3, 96.3, and 98.0% of the total FAMEs detected in the oxic, intermittent, and anoxic soils, respectively (Table 3). Of these 41 FAMEs, 16:0 represented >10% of the total extracted FAMEs in all soils tested, and its percentage increased with increasing soil depth and oxygen limitation. On the other hand, 16:1ω5c also represented >10% of total extracted FAME in the oxic soil, but percentages of this FAME decreased with increasing soil depth and oxygen limitation. Percentages of some FAMEs were not significantly different among soils of different depth and oxic states. Two combined peaks, i 16:1/14:0 3OH and i 17:1 I/a 17:1 B, had relative abundance significantly higher in soils from the intermittent layer than the oxic or anoxic layers.

Using individual FAMEs as biomarkers, the relative abundance of specific microbial groups was compared among the soils (Table 4). The protozoa indicator (20:4ω6c) was

Table 2 – Total number and peak area of FAME detected in soils of redox state specified[a]

Soil[b]	Oxic		Intermittent		Anoxic	
	Number	Area	Number	Area	Number	Area
DS1	$59^{b}{}_{A}$	$1{,}402{,}417^{a}{}_{A}$	$47^{b}{}_{B}$	$216{,}798^{b}{}_{B}$	$34^{c}{}_{C}$	$78{,}188^{b}{}_{C}$
DS2	$53^{e}{}_{A}$	$571{,}658^{b}{}_{A}$	$45^{c}{}_{B}$	$151{,}468^{c}{}_{B}$	$31^{d}{}_{C}$	$44{,}786^{cd}{}_{C}$
DS3	$55^{cd}{}_{A}$	$543{,}269^{cb}{}_{A}$	$53^{a}{}_{A}$	$343{,}504^{a}{}_{B}$	$39^{a}{}_{B}$	$127{,}794^{a}{}_{B}$
DS4	$55^{cd}{}_{A}$	$284{,}318^{d}{}_{A}$	$23^{f}{}_{B}$	$28{,}370^{d}{}_{B}$	$13^{f}{}_{C}$	$18{,}829^{d}{}_{C}$
US1	$64^{a}{}_{A}$	$586{,}318^{b}{}_{A}$	$39^{d}{}_{B}$	$121{,}443^{c}{}_{B}$	$37^{b}{}_{B}$	$53{,}726^{bc}{}_{C}$
US2	$56^{c}{}_{A}$	$518{,}076^{bcd}{}_{A}$	$33^{e}{}_{B}$	$66{,}713^{d}{}_{B}$	$31^{d}{}_{B}$	$39{,}714^{cd}{}_{B}$
US3	$64^{a}{}_{A}$	$503{,}781^{bcd}{}_{A}$	$54^{a}{}_{B}$	$163{,}397^{c}{}_{B}$	ND	ND
US4	$54^{cd}{}_{A}$	$297{,}964^{cd}{}_{A}$	$23^{f}{}_{B}$	$29{,}399^{d}{}_{B}$	$15^{e}{}_{C}$	$20{,}375^{d}{}_{B}$

[a] Different letters indicate significantly different means at $P < 0.05$ according to least sigificiant difference test, where small letters (a–c) indicate comparisons within a column, and capital letters (A–C) indicate comparisons within a row. ND = not determined.
[b] D = Downstream location, U = Upstream location, and S = site.

Table 3 – Soil FAMEs (mean ± S.E., $n = 8$ for oxic and intermittent soils and $n = 7$ for anoxic soils, excluding FAMEs that were <0.5% in all three layers)

FAME	% of total extracted FAME in soils		
	Oxic	Intermittent	Anoxic
10:00	0.46 ± 0.11	0.80 ± 0.16	0.74 ± 0.20
10:0 3OH	0.45 ± 0.08	0.57 ± 0.10	0.74 ± 0.20
12:00	2.27 ± 0.21	4.70 ± 0.98	7.12 ± 2.04
i 13:0	0.69 ± 0.06	0.65 ± 0.12	0.65 ± 0.18
a 13:0	0.54 ± 0.04	0.66 ± 0.11	0.75 ± 0.24
12:0 3OH	0.66 ± 0.07	0.82 ± 0.13	1.29 ± 0.28
i 14:0	1.02 ± 0.06	0.95 ± 0.11	0.97 ± 0.26
14:0	2.76 ± 0.16	4.34 ± 0.33	6.50 ± 0.81
i 15:0	4.02 ± 0.23	4.31 ± 0.19	4.75 ± 0.49
a 15:0	3.18 ± 0.20	3.98 ± 0.10	4.62 ± 0.34
15:1ω8c	0.19 ± 0.04	0.54 ± 0.30	ND
15:0	0.76 ± 0.04	1.03 ± 0.09	1.17 ± 0.18
16:0 N alcohol	1.13 ± 0.12	1.11 ± 0.15	1.39 ± 0.35
i 16:0	2.23 ± 0.25	2.13 ± 0.11	1.90 ± 0.36
16:1ω5c	11.60 ± 1.54	6.03 ± 1.14	2.61 ± 0.85
16:0	12.55 ± 0.76	15.96 ± 2.75	20.17 ± 1.73
15:0 2OH	0.61 ± 0.08	0.20 ± 0.14	0.19 ± 0.19
10Me16:0	2.94 ± 0.21	2.89 ± 0.32	2.36 ± 0.62
i 17:0	0.96 ± 0.10	1.02 ± 0.18	1.36 ± 0.36
a 17:0	0.95 ± 0.09	1.36 ± 0.07	1.11 ± 0.29
17:1ω8c	0.92 ± 0.53	ND	ND
17:1ω7c	0.87 ± 0.22	1.80 ± 0.59	1.78 ± 0.27
cy 17:0	0.95 ± 0.09	0.42 ± 0.14	0.13 ± 0.13
16:1 2OH	0.88 ± 0.13	0.83 ± 0.21	0.88 ± 0.31
16:0 2OH	0.76 ± 0.05	0.56 ± 0.14	0.16 ± 0.16
10 Me17:0	0.50 ± 0.04	0.16 ± 0.05	0.03 ± 0.03
16:0 3OH	0.98 ± 0.08	1.67 ± 0.12	3.14 ± 0.27
18:3ω6c (6,9,12)	1.09 ± 0.13	1.98 ± 0.46	1.58 ± 0.37
18:1ω9c	7.98 ± 0.95	5.08 ± 0.54	3.13 ± 0.62
18:1ω7c	4.09 ± 0.63	2.65 ± 0.52	1.63 ± 0.45
18:0	2.01 ± 0.31	5.20 ± 1.25	6.63 ± 1.19
i 17:0 3OH	0.83 ± 0.09	0.59 ± 0.14	0.70 ± 0.21
cy 19:0ω8c	0.78 ± 0.08	0.47 ± 0.11	0.28 ± 0.14
20:4 ω6,9,12,15c	0.79 ± 0.07	ND	ND
18:0 3OH	0.67 ± 0.05	0.59 ± 0.15	1.32 ± 0.28
18:0	0.63 ± 0.17	1.84 ± 0.50	4.48 ± 2.40
18:2ω6c	4.21 ± 0.32	2.94 ± 0.55	1.29 ± 0.26
i 16:1 I/14:0 3OH	1.77 ± 0.29	5.71 ± 2.28	2.25 ± 0.45
16:1ω7c/*i* 15:0 2OH	6.18 ± 0.41	3.76 ± 0.88	3.22 ± 1.07
i 17:1 I/*a* 17:1 B	0.18 ± 0.13	1.28 ± 0.63	ND
19:1ω6c/unknown/cy19:0ω10c	5.24 ± 0.92	4.69 ± 0.67	4.98 ± 0.55

Table 4 – Percentage of microbial indicator FAMEs in total detected FAMEs in soils tested (mean ± S.E., $n = 8$ for oxic and intermittent soils and $n = 7$ for anoxic soils, ND = not detected)

Microbial group/FAME	Oxic (%)	Intermittent (%)	Anoxic (%)
Protozoa			
20:4ω6c	0.79 ± 0.19	ND	ND
Fungi			
16:1ω5c	11.60 ± 4.37	6.03 ± 3.80	2.61 ± 0.79
18:1ω9c	7.98 ± 2.68	5.80 ± 1.25	3.65 ± 0.78
18:2ω6c	4.21 ± 0.90	2.87 ± 1.70	1.70 ± 0.52
18:3ω6c	1.09 ± 0.36	1.98 ± 1.31	1.58 ± 0.97
G^+ bacteria			
15:0	0.76 ± 0.12	1.03 ± 0.26	1.28 ± 0.44
a15:0	3.18 ± 0.55	3.98 ± 0.27	4.62 ± 0.91
I 15:0	4.02 ± 0.65	4.31 ± 0.53	4.75 ± 1.29
i 16:0	2.23 ± 0.72	2.13 ± 0.30	2.11 ± 0.70
a 17:0	0.95 ± 0.25	1.36 ± 0.21	1.56 ± 0.16
i 17:0	0.96 ± 0.28	1.02 ± 0.51	1.91 ± 0.24
G^- bacteria			
cy 17:0	0.95 ± 0.26	0.62 ± 0.52	ND
18:1ω7c	4.09 ± 1.78	2.65 ± 0.52	1.63 ± 0.45
Actinomycetes			
10Me17:0	0.50 ± 0.11	0.27 ± 0.20	ND
10Me18:0	0.40 ± 0.20	0.21 ± 0.30	ND
10Me16:0	2.94 ± 0.61	2.89 ± 0.89	2.36 ± 1.65
Ratio of total saturated/total mono-unsaturated fatty acids	0.94 ± 0.98	1.37 ± 0.38	3.93 ± 0.48
Ratio of cyclopropyl fatty acids/monoenoic precursors	5.94 ± 1.04	7.37 ± 1.40	11.83 ± 1.52

detected only in the oxic layer. The relative abundance of fungi, actinomycetes, and G^- bacteria indicators were significantly higher in oxic layers when compared with those in anoxic layers. On the contrary, the relative abundance of G^+ bacteria increased with increasing soil depth and oxygen limitation. Similarly, ratios of total saturated/total monounsaturated fatty acids and cyclopropyl fatty acids/monoenoic precursors also increased with increasing soil depth and oxygen limitation (Table 4).

Principal component analysis (PCA) of the FAME indicators showed that the first principal component (PC1) explained 38.2% of the variance while the second (PC2) explained 29.7% for a total of 67.9% of the variability in the data being explained (Fig. 1). The PCA showed that the microbial communities clustered into four distinct groups (labeled as A–D), separating according to redox state and flooding influence. Microbial communities from the site 4 soils in the intermittent (two soils) and anoxic layers (four soils) clustered together (group D), which were apart from soils under the influence of periodic flooding.

3.2. Soil DNA concentrations and T-RFLP analysis of bacterial community

DNA extracted had satisfactory purity, showing A260/A280 ratios ranging from 1.70 to 1.85 (data not shown). The average soil DNA concentrations (±standard error) were 6.7 ± 1.1 $\mu g\, g^{-1}$, 3.5 ± 0.6 $\mu g\, g^{-1}$, and 1.5 ± 0.2 $\mu g\, g^{-1}$ soil for the oxic, intermittent, and anoxic soils, respectively. By average, the amount of DNA recovered from each gram of oxic soil was 4.5-fold of that recovered from the anoxic soil.

Among the T-RFLP fingerprints obtained, terminal fragments (TRF) in the size range of 50–700 bp were clearly distinguishable. Typical T-RFLP fingerprints are shown in Fig. 2. The richness of detected ribotypes in each redox state was estimated by the total number of terminal restriction fragments (TRFs) (Table 5). Among the five enzymes used, HaeIII, AluI, HhaI and HpaI revealed more ribotypes, while RsaI revealed the least number of ribotypes (data not shown). The mean TRFs number estimated from five restriction enzymes was highest in the oxic layer, followed by that in the intermittent soils, and was the least in the anoxic soils (Table 5). The average number of TRFs in the oxic soils was twice of that in the anoxic soils.

Bacterial diversity as indicated by the Shannon index (H′) was significantly higher in the oxic layers than in the intermittent or anoxic layers (Table 5). However, the Pielous's indices for distribution evenness (J) of the bacterial community were not significantly different among soils of different redox states, which were 0.82 ± 0.04, 0.82 ± 0.03, and 0.80 ± 0.02 for the oxic, intermittent, and anoxic layers, respectively ($n = 105$–120, $p < 0.05$). The distribution evenness of a community can also be indicated by dominance of individual ribotypes in a community, as reflected by the relative band intensities on a T-RFLP fingerprint. The higher the intensity, the more dominant is the bacterial ribotype corresponding to that band. Based on the intensity of the three most intense bands in each community, expressed as percentages of total DNA intensity detected in the lane, the most even distribution of bacterial ribotypes occurred in the oxic layer, with the three most intense ribotypes comprising about 32% of the total bacterial community detected. Bacterial

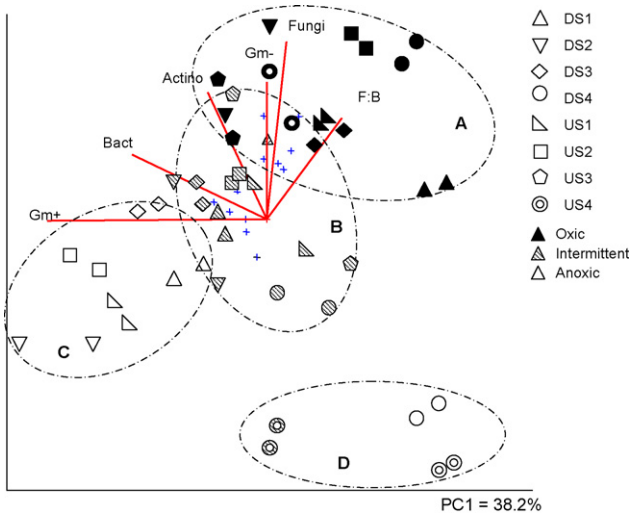

Fig. 1 – Principal component analyses (PCA) of fatty acid methyl ester (FAME) in soils from oxic (A), intermittent (B), and anoxic layers (C). Two replicate analysis are presented. Indicator FAMEs are bacteria (15:0, *a* 15:0, *i* 15:0, *i* 16:0, *a* 17:0, *i* 17:0, cy17:0, and 18:1ω7c), actinomycetes (10Me16:0, 10Me17:0, and 10Me18:0), and fungi (16:1ω5c, 18:1ω9c, 18:2ω6c, and 18:3ω6c). The PCA was performed using cross-products matrix with variance/covariance centered and calculating scores for FAMEs by weighted averaging. Comparison among soils is based on the predominance of the microbial groups as shown by their grouping close to vector lines designated for fungi and bacteria (Bact: Gm^+, Gm^-, Actino (actinomycetes)), and for fungi:bacteria ratio (F:B). Microbial communities from two intermittent and four anoxic layers of the site 4 soils were clustered together (D) and were distinctively different from those in soils under the influence of flooding. DS1, DS2, DS3, DS4, US1, US2, US3, and US4, where D = downstream, U = upstream, and S = site.

communities in the anoxic soils were the least evenly distributed (Table 5).

Similarity of bacterial communities in the soils tested was evaluated by pair-wise comparisons based on band sharing coefficients. A total of 253 band sharing coefficients were obtained. In general, band sharing coefficients were greater than 0.5 between bacterial communities in oxic layers when compared within each location or between the two locations (Table 6). However, bacterial communities among the intermittent soils shared little similarity, showing that 10 out of 28 pairs had greater than 0.5 band sharing coefficient. Bacterial communities among the anoxic soils shared even less similarity, showing that only 2 out of 21 pairs had band sharing coefficients greater than 0.5. The difference among bacterial communities between different redox states was substantial, showing only 2 out 176 pair with band sharing coefficients greater than 0.5.

Similarity of bacterial communities was further revealed by cluster analysis of dendrograms based on T-RFLP fingerprints. Bacterial communities were clustered based on soil redox states as there were three major clusters corresponding to oxic, intermittent, and anoxic layer soils, respectively (Fig. 3).

4. Discussion

The distinct grouping of subsurface microbial communities according to flooding recurrence intervals (Fig. 1) based on FAME analysis suggested a dominant influence of flooding in shaping subsurface microbial diversity, composition, and community structure. This was also evidenced by the total number and abundance of FAME detected (Table 2). However, distinct grouping of microbial communities according to flooding influence was not observed for the oxic soils irrespective of great influence of periodic flooding and environmental contamination. This finding is consistent with those reported by other investigators (Konstantinidis et al., 2003; Zhou et al., 2002). Although metabolic processes and specific groups of bacteria could be sensitive to environmental perturbation (Sandaa et al., 1999), the overall microbial community could be resilient to changes in the environment, especially for the highly diverse microbial communities in the surface oxic layers.

Because of wide variations in environmental conditions and periodic flooding in the surface soils, one might expect wider variations in microbial communities between surface soils than between subsurface soils. Data obtained from this study were not consistent with previous reports which were also based on analysis of fatty acid composition and 16S rRNA genes (PCR-denaturing gradient gel electrophoresis) (Fritze et al., 2000; Goberna et al., 2005). The discrepancies among studies could be attributed to sampling strategy. Most studies reported data obtained from soils sampled from different depths irrespective of redox state, a predominant factor regulating bacterial diversity and community structure. The increased variations in bacterial diversity and community structure with decreasing oxygen supply in this study suggested emergence of other stress factors. For example, the nutritional stress indicator in the anoxic soils was twofold to fourfold of that in the oxic soils (Table 4).

It has long been recognized that organic C content decreases with increasing soil depth, accompanied by a decrease in microbial biomass (Anderson and Domsch, 1989; Agnelli et al., 2004). Analyses based on FAME, soil DNA concentration, and 16S rRNA fingerprinting of microbial communities showed that the deeper intermittent and anoxic soils had significantly lower microbial abundance and diversity than the surface oxic soils. The change in microbial abundance was greater than the change in microbial diversity, suggesting that growth of the microbial community is more energy demanding than maintenance. Killham and Firestone (1984) showed that the microbial community increased its basal respiration under stress conditions. Under oxygen limiting conditions, the microbial community had low energy yield from metabolizing reduced substrates, leading to low C use efficiency. In another related study (Unpublished data), we found that microbial biomass C accounted for 2.28, 1.79, and 0.99% of soil organic C in oxic, intermittent, and anoxic soils, respectively. Oxygen limitation can lead to elimination of

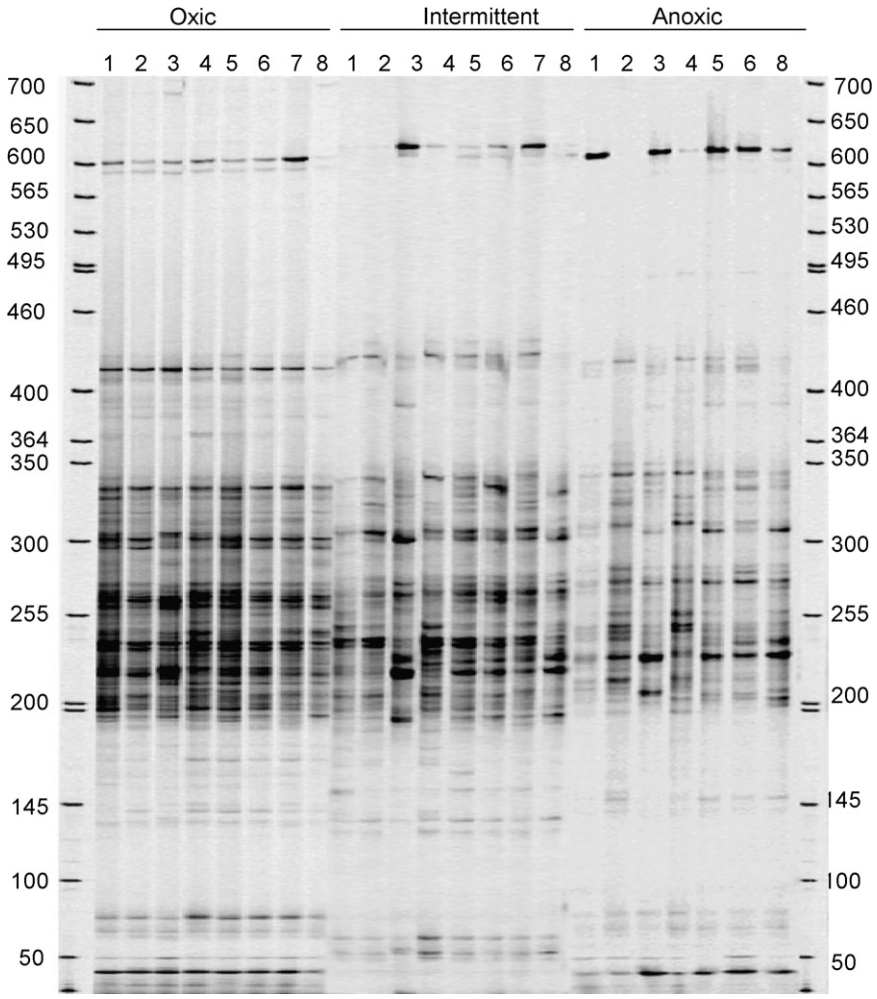

Fig. 2 – 16S rRNA gene fingerprints of soil bacterial communities obtained by soil community DNA extraction, PCR amplification of an internal region (927 bp) of 16S rRNA genes in the bacterial domain, HaeIII digestion, and terminal-restriction fragment length polymorphism (T-RFLP) separation. For each redox layer, samples 1–8 are in the order of DS1, DS2, DS3, DS4, US1, US2, US3, and US4,.where D = downstream, U = upstream, and S = site. DNA markers ranged from 50 to 700 base pair (bp).

aerobic microbial groups, changes in microbial community structure, and reduced microbial diversity, as evidenced by changes in FAME composition, relative abundance of different FAME indicators, as well as the number of detected T-RFLP bands and banding patterns. Direct linkage between bacterial diversity and oxygen supply has been reported in other studies (Liu et al., 1997; Urakawa et al., 2000). Low diversity at deep soil layers is also consistent with species–energy theory, which predicted relatively low diversity in the low organic matter horizon (Wright, 1983).

Table 5 – Soil DNA concentrations and analysis of soil bacterial community by T-RFLP fingerprinting PCR-16S rRNA genes[a]

Soil redox state	Total number of TRFs	Shannon diversity index (H′)[b]	The intensity of three most intense bands in each community expressed as percentages of total DNA intensity in the lane
Oxic	41 ± 0.6	1.32 ± 0.03	32.13 ± 2.6
Intermittent	24 ± 1.0	1.13 ± 0.02	37.17 ± 2.3
Anoxic	21 ± 1.3	1.04 ± 0.02	41.60 ± 2.3

[a] Data shown are means obtained from three replicate T-RFLP fingerprints with five different restriction enzyme digestions of PCR-16S rRNA genes using template DNA from 8 oxic or intermittent layer soils or 7 anoxic layer soils. Thus, for the analysis of soil bacterial community fingerprints, $n = 120$ for oxic or intermittent soils, and $n = 105$ for anoxic soils.

[b] $H' = -\Sigma(\%\text{IntOD}) \times \log(\%\text{IntOD})$, where %IntOD represented the integrated DNA density of a band, relative to the sum intensity of all the bands in a lane.

Table 6 – Number of sampling sites within each location or between two locations that had greater than 0.5 similarity coefficient between paired terminal restriction fragment length polymorphism (T-RFLP) fingerprints[a]

Paired redox states[b]	Location comparision			
	Within D	Within U	D with U	Sum
OX with OX	6/6	6/6	15/16	27/28
Int with Int	3/6	5/6	2/16	10/28
AX with AX	0/6	1/3	2/12	3/21
OX with AX	0/16	2/16	0/32	2/64
OX with Int	0/16	0/12	0/28	0/56
Int with AX	0/16	0/12	0/28	0/56

[a] A total of 253 band sharing coefficents were obtained; 28 were within the oxic layers, 28 within the intermittent layers, 21 within the anoxic layers, and 176 between layers of different redox states.
[b] D = Downstream location, U = Upstream location, OX = oxic, Int = intermittent, and AX = anoxic.

Although total microbial abundance and diversity decreased with increasing soil depth, the relative abundance of some FAME indicators, such as FAME 16:0, and the intensity of some T-RFLP bands (Fig. 2) were considerably higher in the subsurface intermittent and anoxic soils than in the oxic soils. FAME 16:0 has been used as an indicator of nutritional stress in bacterial communities (Kieft et al., 1997; Fierer et al., 2003). The increase of this FAME from 13 to 20% when shifting from oxic to anoxic environment reflected changes of membrane fatty acids with respect to maintenance of membrane fluidity (Guckert et al., 1986). The significantly higher abundance of G^+ bacterial indicators in the anoxic layers than the oxic layers (Table 4) is consistent with reports that their abundance increases with increasing soil depth (Fierer et al., 2003). As a dominant microbial group in all soils tested, growth and maintenance of G^+ bacteria were apparently not as sensitive to oxygen and C supplies when compared with other microbes. Sinclair and Ghiorse (1989) reported that G^+ bacteria were associated with high clay content while G^- bacteria were associated with sandy sediments, a correlation that was not supported by this study (data not shown). The dominance of G^+ bacterial abundance in the reduced soil environment was often accompanied by a low protozoan population (England et al., 1993; Sinclair and Ghiorse, 1989). The strictly aerobic protozoans were detected only in the oxic soils, indicating that FAME analysis was discriminating in the detection of protozoans.

The dominance of FAME 16:1ω5c (>10% abundance in oxic layers) was of ecological significance because this FAME has been suggested as an indicator of mycorrhiza in soils and its relative abundance has been correlated to the activities of phosphatases (Olsson, 1999; Acosta-Martínez et al., 2007). A study using C isotope tracking showed that mycorrhizal fungi played an important role in facilitating the flow of C from the above-ground plant community to the below-ground soil microbial community (Krsek and Wellington, 2006). Madan et al. (2002) reported that 16:1ω5c was the dominant fatty acid present in several fungal species of endomycorrhizal associations, including *Glomus coronatum*, *Glomus mosseae*, *Gigaspora margarita* and *Scutellospora calospora*. The detectable presence of this FAME indicator at 2.61% by average in the deep anoxic

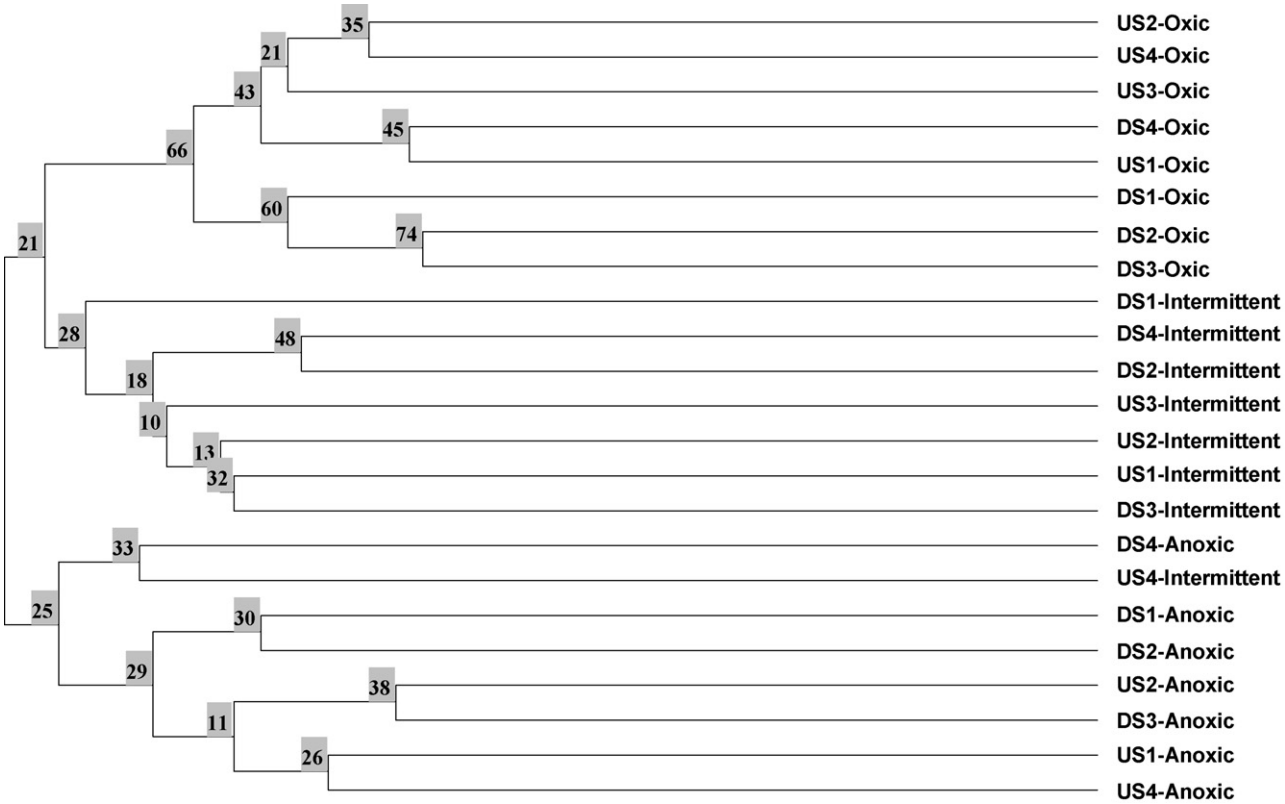

Fig. 3 – A typical dendrogram generated from a T-RFLP fingerprint. This dendrogram was generated with HaeIII digestion and constructed from "0/1" string data at 0.5% match tolerance.

soils (Table 3), however, suggests that this FAME indicator may not be specific to aerobic fungal species that are capable of forming endomycorrhizal associations with plant roots.

It is interesting that the combined peaks, i16:1/14:0 3OH and i17:1 I/a17:1 B, had significantly higher relative abundance in soils from the intermittent layer than the oxic or anoxic layers. OH fatty acids (usually 3 OH) are often found in G^- bacteria, while branched fatty acids (iso, anteiso) are more commonly associated with G^+ bacteria but are also found in G^- bacteria (Cavigelli et al., 1995; Fierer et al., 2003). Additional research will be required to determine whether these FAME peaks indicate relative abundance of facultative bacteria or reflect more general changes in membrane fatty acid composition corresponding to bacterial adaptation to an alternating oxic-reducing environment.

Not only did the abundance of specific microbial groups, bacterial diversity, and distribution evenness vary along soil profiles (Fritze et al., 2000; Urakawa et al., 2000; Agnelli et al., 2004; this study), but also microbial species in the subsurface soils could be strikingly different from those in the surface soil (Crocker et al., 2000; Fritze et al., 2000; Blume et al., 2002). Our data further supported this observation, showing that microbial community structures of the intermittent and anoxic layers shared more similarity with each other, but little with those in the oxic layers. It is evident that redox state, and C quantity, quality, and availability are all important factors regulating the bacterial communities in the soil environment (Kieft et al., 1994; Sahm et al., 1999, Fierer et al., 2003; this study). The challenge is to identify the key factor that is of dominant influence. The distinct clustering of microbial groups according to soil redox state as shown by multivariate analyses for the FAME (Fig. 1) and by the cluster analysis of T-RFLP fingerprints (Fig. 3) suggests that oxygen played a dominant role driving the changes in microbial community structure and composition. Although organic C contents of soils tested varied considerably with a general trend of decreasing with increasing soil depth (Unpublished data), the microbial community did not cluster according to soil organic C contents.

FAME analysis presumably revealed changes in the entire microbial community, while T-RFLP revealed changes only in the bacterial community because the PCR primers were selected to amplify 16S rRNA genes of the bacteria domain. The generally close agreement between these two techniques on the detected differences in microbial community structure and diversity in this study suggested that bacteria play a more dominant role than other microbial groups in shaping microbial community structure and diversity in the soil ecosystem.

5. Conclusion

Redox state played a dominant role regulating microbial growth, diversity, and community structure, leading to development of subsurface microbial communities exhibiting little resemblance to those of the surface soils. Bacteria are dominant components of soil microbial communities, while mycorrhizal fungi are an important group in the oxic soils. Gram-positive bacteria were relatively insensitive to oxygen and organic carbon supplies and their relative abundance increased by about 33% when soils shifted from the oxic to anoxic conditions even though soil organic C contents decreased from as high as 2.99 in the oxic soils to as low as 0.29% in the anoxic soils.

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